Exhibit A

Reference 1

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バナナ（*Musa acuminata*）レクチンによる多糖の内部鎖の特異的認識 ―グルカン分子中のα-1,3およびβ-1,6グルコシド結合の認識―

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Novel Recognition by Banana (*Musa acuminata*) Lectin of Internal α-1,3 and β-1,6-linked D-Glucosyl Residues

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Summary

A new α-Man/Glc binding lectin, designated BanLec, was isolated from banana (*Musa acuminata*) fruits by Koshte *et.al* (1990). The extensive study by Peumans *et al* (2000) indicated that the lectin is present in the pulp of ripe fruits, and related to that from plantain (*Musa* spp). Goldstein and coworkers (2001) found unexpectedly that this lectin binds not only branched α-mannan and glucan but also linear α-glucans, such as nigeran and elsinan by recognizing their internal 1,3-glucosidic linkages. It also appeared that BanLec binds to some *O*-6-branched β-glucans. In the present study the lectin was newly isolated by affinity column of α-1,3-glucan or branched *Auricularia* β-glucan.; the lectin was dimer of 14 kDa protein. The binding capability of BanLec was confirmed by use of the lectin-conjugated affinity column. Among various linear α-glucans, nigeran (1,3/1,6) and elsinan (1,3/1,4), but pullulan (1,6/1,4) was not able to bind. Interestingly BanLec was found to recognize *Agaricus* β-1,6-glucan, and other β-1,6-glucans, *e.g.*, pustulan and *Gyrophora* glucan, but not β-1,3-glucans, such as curdlan. Thus, the banana lectin was proved to be a unique lectin, recognizing the specific internal linkages of α- and β-glucans.

レクチンは，植物，動物組織や微生物細胞に至るまで，広く自然界に分布し，多糖や生体組織中の複合糖鎖を特異的に認識結合する。生体細胞の認識，免疫反応を含めてレクチンの生体機能における役割は益々重要視されている。自然界で最初に発見された植物レクチンとしては糖鎖末端のα-Glc/Manを認識するコンカナバリンA（ConA）が代表的なものであるが，単子葉植物の球根にはα-Manのみを認識するレクチンがあり，その特異性については詳しい研究がある。Koshteら（1990）は，バナナ（*Musa acuminata*）果実のhomogenateからマンノ・オリゴ糖と結合するレクチン（BanLec）をSephadex columnを用いて分離した。このレクチンは13kDaの蛋白の二量体でヒトのIgG4に結合する事を報告している[1)]。

その後Peumansら（2000）[2)]は，このレクチンが完熟したバナナの果実のパルプ中に存在すること，その抽出物をmannose-affinity columnに吸着させ，α-メチル-マンノシドでeluteすることに依って15kDaの蛋白の二量体であるこ

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と，結合性はα-Man＞Glc,さらに，クローニングによりJacalin種のレクチンと同じような三次元構造をもつ事を明らかにしている。

レクチンの糖鎖認識特異性はConAのように基本的には多糖や複合糖鎖の末端の糖残基を認識する。しかし，最近我々が報告したように，単子葉植物のα-Man結合レクチンのなかには*Crocus*球根のα-Man結合レクチン[3]，また，*Listeria ovata*の葉から分離したレクチンのなかにはα-マンナンの糖鎖中の1,3結合を認識するものもある[4]。

Peumansらの研究に続いてGoldsteinら (2001)[5, 6]はBanLecの糖鎖結合特異性の研究を行ったが，定量沈降反応および結合阻害を調べてこのレクチンが酵母のα-マンナン，グリコーゲンとは結合するが，α-1,4-,1,6-結合の直鎖のpullulanには結合しないこと，他方，内部にα-1,3-結合を有する直鎖のnigeranやelsinan (α-1,3/1,4)[7]などと結合することを見出した。さらに彼らは，このレクチンが1,6分岐のβ-1,3グルカンにも結合する事を見出した[6]。このことは，バナナのレクチンがこれまでレクチンの概念を越えたユニークな糖鎖認識性をもつ可能性を示唆した。

これらの研究のさらなる過程で，我々は，バナナのレクチンが*Agaricus blazei*の子実体に含まれる水抽出性のβ-1,6グルカン[8]とも結合する可能性がある事を見出したので，新たにBanLecをリガンドとするaffnity column chromatographyを用いて結合の異なるα-およびβ-グルカンに対する結合機能を明らかにしようと試みた。

実験方法および結果

BanLecの分離精製　新鮮なバナナ (650 g) を潰し，50 mMのPBS (pH 6.5) 中で，数時間攪拌(25℃)後，澱粉など不溶物をチーズクロスで濾過し，濾液を遠心分離後，上清に0.8飽和の硫安を加えた。沈殿した蛋白画分をα-1,3グルカン (*Streptococcus* HHT 由来)，またはキクラゲから得た不溶性のβ-1,6/1,3グルカンのカラムにアプライし，吸着したレクチンを0.2 M α-Me-mannnosideまたは，25 mM diaminopropane (DAP, pH 11) でelute させ，透析後Sephacryl S-200のカラムで精製した (収量，25 mg)。精製レクチンは電気泳動的に均一 (14 kDaのdimer) であった (Fig. 1)。

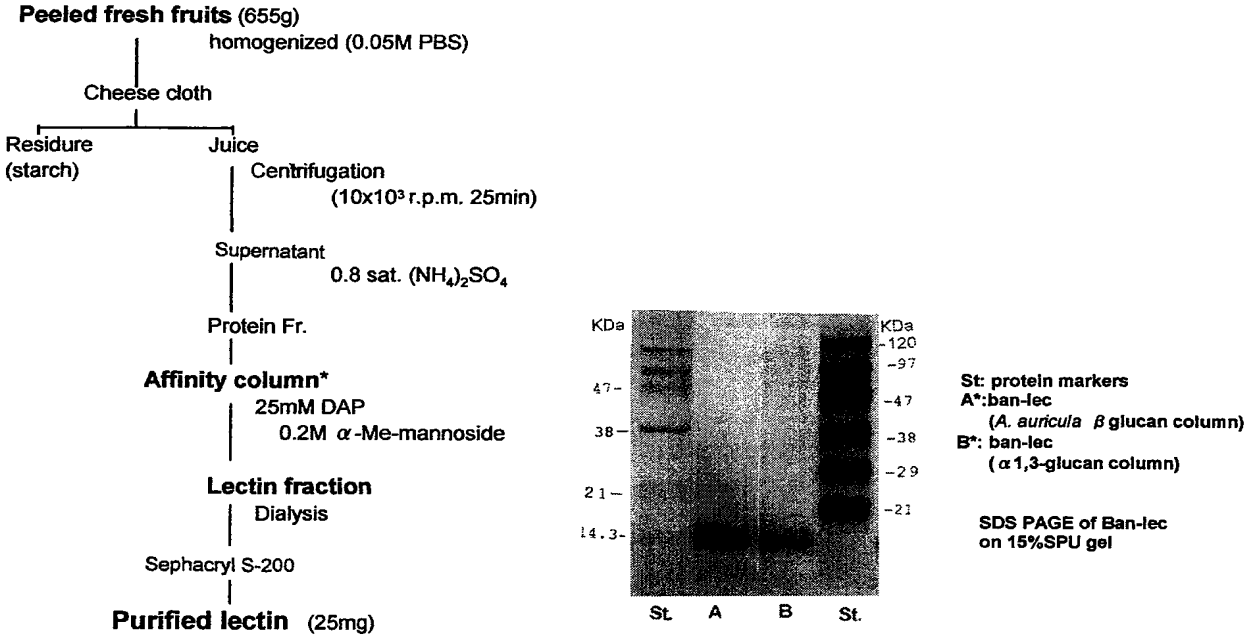


Fig. 1 Fractionation and purification of Banana lectin (BanLec).

定量沈降反応　一般的には1.5 ml容のcentrifuge tube中でレクチン25-30 μg,に多糖，またはオリゴ糖（10-120 μg）を50 mMのPBS（pH 6.8）150 μl中で反応（10℃, 48 hr）させ，沈降蛋白をmicro Lowry法で定量（750 nm）した。その結果はFig. 2およびFig. 3に示す如くα-グルカンのなかでは，我々が見出した*Elsinoe leucospila*の生産するelsinan（1,3/1,4直鎖）とよく反応する。Fig. 4に示すごとく，このグルカンはBanLecと特異的に反応するが，ConAとは本質的に反応しない。この事実は重要であり，さらに興味あることはα-1,6-と1,3の交互結合をもつ1355 dextranともある程度反応するが，α-1,6/1,4-結合のpullulanとは反応しないことである。また，BanLecは糖鎖末端のα-Manおよびα-Glcに特異的でβ-結合の末端とは結合しないにも関わらず，ある種のβ-結合のグルカン，とくに，β-1,6結合のアガリクスの水溶性グルカンや地衣類の一種イワタケのβ-1,6-richのグルカンとよく反応する。*O*-6分岐結合のβ-1,3グルカンであるschizophyllanとも反応するが，枝のグルコシル基を還元したschizophyllan polyolとは反応しない。このことは，β-1,3グルカンを認識出来ないことを示す。BanLecはβ-1,6結合のgentioheptaoseとも反応するので，このレクチンはβ-1,6結合の分枝に特異性をもつというよりむしろ，β-1,6の結合鎖を認識すると考えられる。この点については更に明確にしたい。

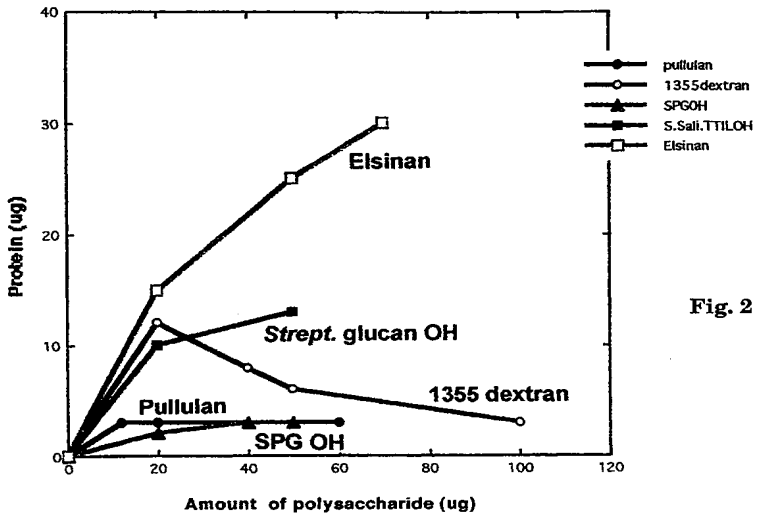


Fig. 2 Quantitative precipitation curves of BanLec with some α-glucans (elsinan, 1355 dextran, pullulan) and glucan polyol of *Streptococcus salivarius* branched 1,3-glucan and schizophyllan polyol.

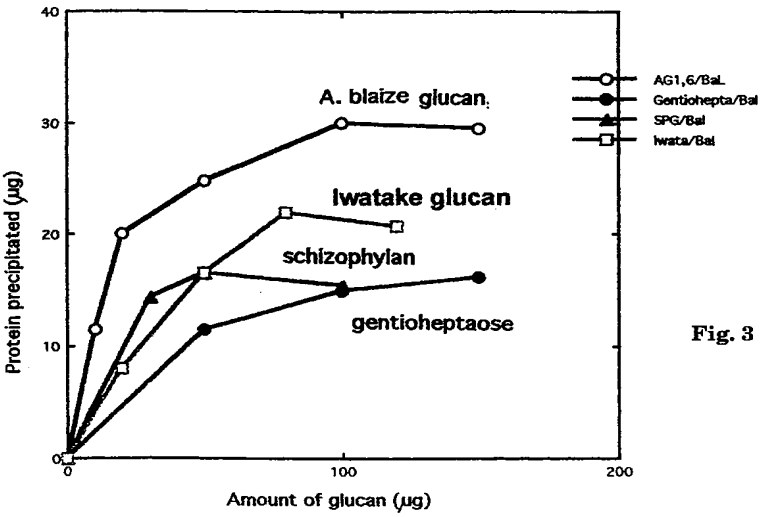


Fig. 3 Quantitative precipitation curves of BanLec with β-glucans of *Agaricus blazei*, Iwatake, schizophyllan and gentioheptaose.

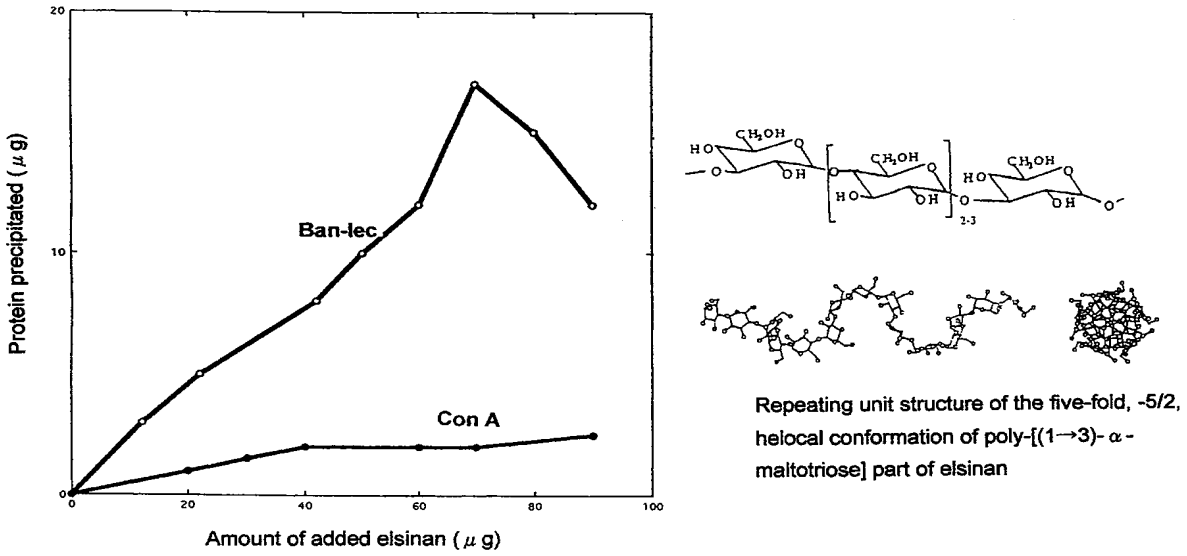


Fig. 4 Quantitative precipitation curves of elsinan with BanLec and ConA (Note: ref. 5).

BanLecのaffinity columnの作成と多糖のchromatography *Crocus*球根のα-Man結合レクチンに準じ，BanLec (15 mg) をAF-tresyl Toyopearl (Toso Co: 650 mg) と100 mM PBS (pH 8.0) 中で25℃，6 hr，次いで10℃，4 hr，緩やかな攪拌によって結合させる。未反応のtresyl groupを100 mM Tris-HCl buffer (pH 8.0) でblockingした。これにより約65%のレクチンがconjugateされていた。このBanLecのlectin conjugateを小カラムに充填 (1×6 cm) したものをaffinity columnとした。このカラムによる多糖の分離としては酵母マンナンの他，構造の明確なα-およびβ-グルカンまたはその混合物 (0.5-1 mg) をPBSに溶かして，カラムにアプライし，最初はPBSで非吸着画分を流し出した後，20-25 mMのDAPでretainした多糖をeluteさせる。流出液は通常0.5-0.75 mlずつ分取し，糖量はフェノール硫酸試薬で比色定量 (490 nm) した。

Fig. 5にはこれら多糖のaffinity chromatographyによる分離結果の例を示した。BanLecは糖鎖末端のα-Manに特異的で，そのため酵母の分岐マンナンはBanLec columnにretainする。これはaffinity columnの有効性を示す。

このレクチンは分岐構造の*α*-グルカンであるglycogenと結合するが，α-1,6/1,4-結合のpullulanはretain出来ない。これとは対照的に内部に1,3結合をもつelsinanは強くretainした。このレクチンはα-1,3結合のnigeran columnに吸着する事実と符合する。β-結合のグルカンのうち6位に分岐をもつ1,3結合のschizophyllanは弱く吸着する。

これに反して*Agaricus*の子実体グルカンのうち水抽出性の精製β-1,6結合の，本質的に非分岐グルカン (ConA columnの非吸着画分) は強くretainされる。なお直鎖のβ-1,3グルカンであるcurdlan (不溶性) には吸着されなかった (Fig. 6)。

このことはBanLecがα-1,3-グルカンには特異的に結合するが，β-1,3-グルカンには結合出来ないという結果を示す。

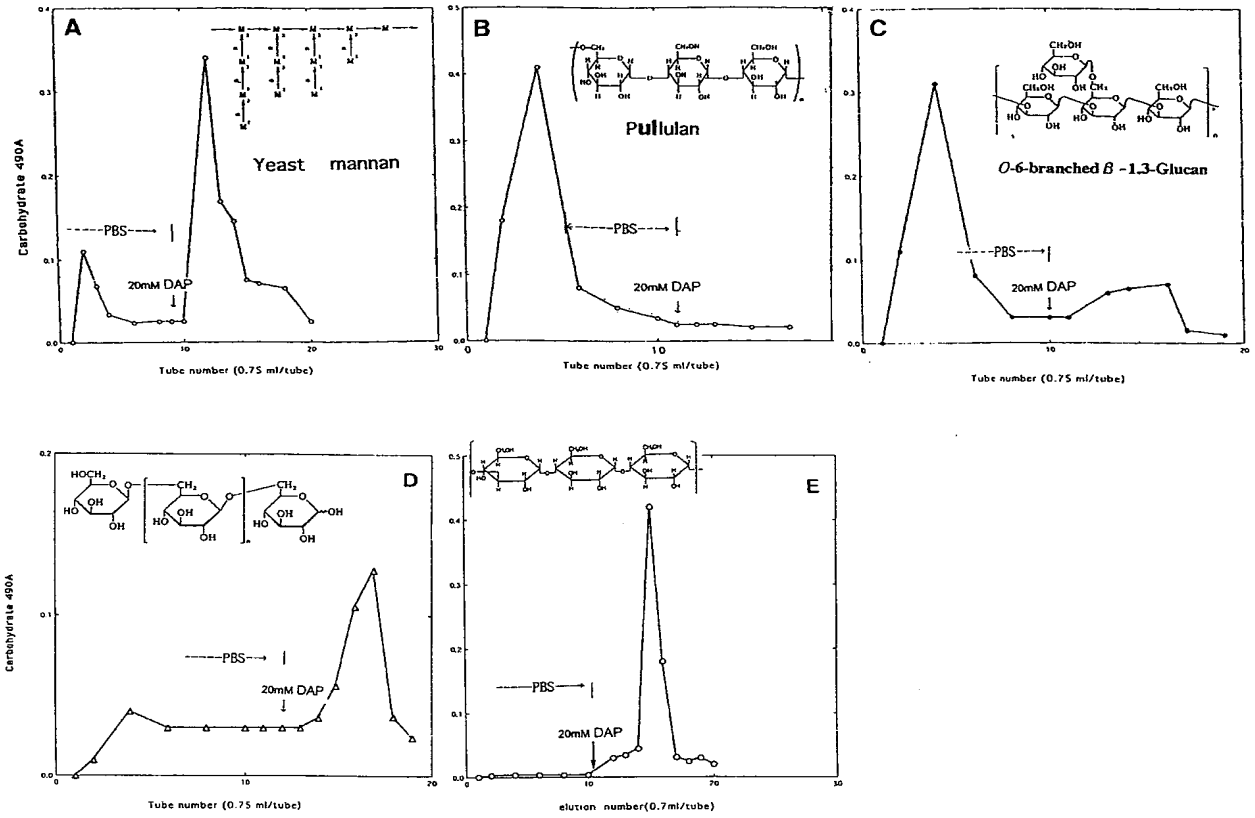


Fig. 5 Elution profiles of some polysaccharides from BanLec affinity column
A: yeast mannan, B: pullulan, C: schizophyllan, D: *Agaricus* β-1,6-glucan E: elsinan
Each 0.5-1 mg of glycan was applied on BanLec column (1 x 5.5 cm); after not-retaining fraction was removed with PBS, then the retaining glycan was eluted with 25 mM DAP.

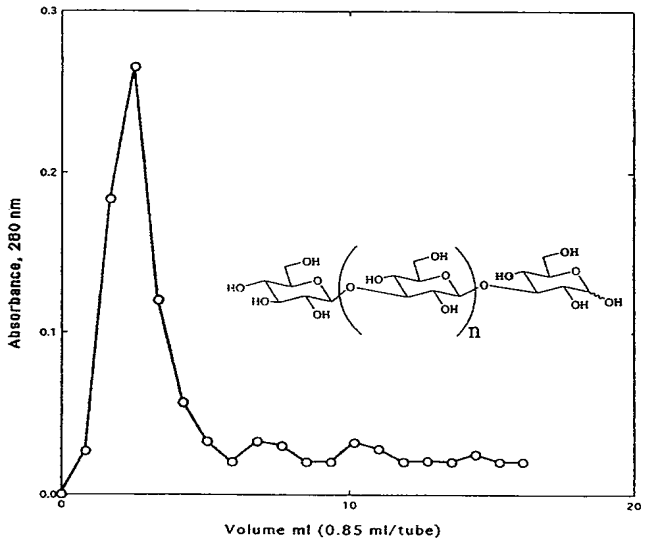


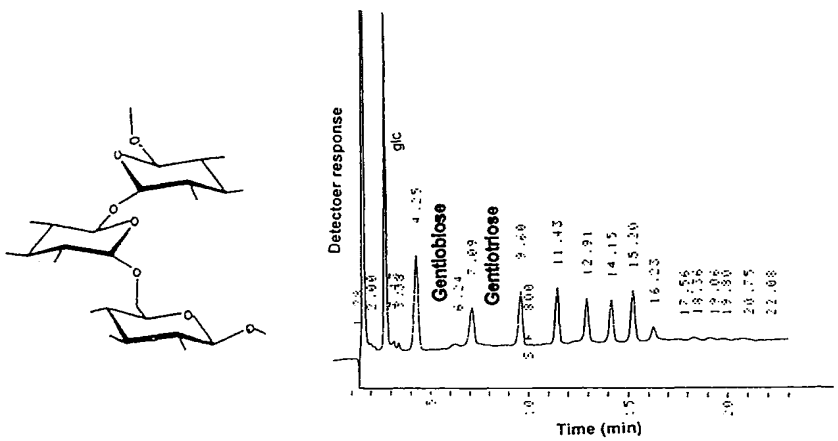
Fig. 6 Elution profile of BanLec (1.5 mg) from curdlan column (0.6 x 5 cm)
Most lectin was eluted with 50 mM PBS, and minor fraction was eluted with 100 mM α-Me-mannoside.

考　察

19世紀の終わりに豆科植物の種子中から発見されたヒト赤血球の凝集素（フィトヘマグルチニン）とよばれたレクチンは，その後，植物，動物および微生物から新しい特異性をもつものが発掘され，現在，糖鎖科学・工学の分野で重要な情報および機能解析の手段となっている。植物レクチンのうち，α-Glc/Manを特異的に認識するものとしてはConAで代表されるように，主として豆科の種子から分離されてきた。しかし，KoshteおよびPeumansら（1990, 2000）はバナナ（*Musa acuminata*）の果実中にもα-Manに特異的で，かつ，強いT-細胞の刺激活性をもつ分子量約30万のレクチン（BanLec）を分離精製した。また，このレクチンは完熟したバナナ果実のパルプ中に存在し，mannose columnやSephadex columnによって分離しうること，また，バナナを含め広くplantain系植物に広く存在することを示唆している。我々はBanLecを不溶性のα-1,3-グルカンやキノコの1,6-分枝をもったβ-1,3-グルカンの小カラムに吸着させることに依って，比較的簡単に電気泳動的に均一なレクチン（14 kDaの蛋白の二量体）を得た。

BanLecの糖鎖結合特異性については，当初，これまでのレクチンと同様に多糖の末端糖鎖に限定されるものと考えられていたが，種々の多糖，オリゴ糖と定量沈降反応で調べてみると，ある種の直鎖型のグルカン，特に，内部にα-1,3-結合の糖残基をもつnigeranやelsinan[7, 9]（Fig. 7）と強く反応した。このことはBanLecが*Streptococcus*のα-1,3-グルカンのカラムに吸着する事実[4]と一致する。しかし，α-1,4-,1,6-結合の直鎖のpullulanには結合しないことが分かった。さらに，本レクチンはβ-1,6グルコシド結合のオリゴ糖およびそのポリマーを特異的に認識する事実が定量沈降反応の結果から明らかにされた（Fig. 2, Fig. 3）。一方，schizophyllanなど*0*-6分岐をもったβ-1,3-グルカンもある程度BanLecと反応するが，分枝のGlcを修飾したschizophyllan-polyolでは反応しないこと（Fig. 2, 3），さらに，直鎖のβ-1,3グルカンであるcurdlanのカラムには吸着しないことから，分岐β-1,3グルカンの結合は分枝を形成するβ-1,6グルコシル基に依ると考えられる。

本研究における我々の目的の一つは，BanLecをリガンドとするaffinity columnによって*Agaricus blazei*の子実体の水抽出画分に含まれるβ-1,6グルカン[8]を単離し得るかという事であった。このキノコに含まれる主要な多糖成分は水不溶，アルカリ可溶の1,6分岐のβ-グルカンであるが，水抽出物からグリコーゲンをConAカラムに吸着させて除くと抗腫瘍活性を示す，本質的に直鎖構造のβ-1,6グルカン（分子量：20-25万）が得られた。その詳細な構造は化学的解析，C^{13}n.m.r.，さらにβ-1,6-glucanhydoraseによる一連のゲンチオ・オリゴ糖の生成から確認された[8]（Fig. 8）。このグルカンの他，地衣類のpustulanおよびイワタケ（*Gyrophora esculenta Miyoshi*）[10]に含まれる1,6-richのグルカン



HPAEC profile of Con-A column unabsorbed glucan after hydrolysis by β1,6-glucan hydrolase

Fig. 7 *Agaricus blazei* β-1,6-glucan and gentio-oligomers formed by β-1,6-glucanase action.

もBanLecによって認識されたのは最初の例である。なお，定量沈降反応でも7糖（gentioheptaose）が結合する事（Fig. 2)からも支持される。

今後の課題としてBanLecが認識できる最少限の1,6-鎖を確定すべきと考える。上述のように本レクチンは，非還元末端が関与しない特定の内部結合の直鎖のグルカンを認識しうる，これまでのレクチンの概念を越えたユニークな糖鎖認識性をもつ可能性を示唆している。これは恐らく，特定の内部のグルコシル残基で規定されるグルカンのコンホメーションが関与するもと考えられる。将来BanLecのような糖鎖の特定の内部結合を認識する新しいレクチンが開発され，種々のグリカンの分画精製の有用なプローブとして利用される事が期待される。

まとめ

1. Banana fruitsのレクチンは末端のα-Man/Glcに特異的であると想定されていたが，elsinanやnigeranなどの糖鎖内部のα-1,3結合をも認識することが分かったので，特定の構造の多糖のカラムを用いて精製した。(dimeric 14 kDa)
2. 結合の異なる種々のα-およびβ-グルカンとの結合性を定量沈降反応，およびBanLecをリガンドとするカラムにおけるaffinityから比較した。その結果，直鎖のα-グルカン として，1,3結合を含むelsinanやnigeranと強く結合するが，α-1,6結合のpullulanは結合しなかった。
3. β-グルカン では，β-1,6のpustulanと結合するが，β-1,3のcurdlanとは結合しない。しかしschizophyllanなど0-6分岐の1,3グルカンとはある程度の結合性を示した。
4. アガリクス（*Agaricus blazei*）の熱水抽出画分からCon A非結合性のグルカンを分離精製し，これが直鎖に近い構造のβ-1,6グルカンであることを化学的，酵素分解およびBanLecのaffinity column chromatographyで確かめた。
5. 以上の結果から，このレクチンは，特定（α or β）の糖鎖末端基に特異的であるという従来の概念を越えて多糖鎖の特定の内部結合をも認識しうることを示した最初の例であろう。

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Exhibit B

Reference 2

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Purification and Characterization of the α-1,3-Mannosylmannose-recognizing Lectin of *Crocus vernus* Bulbs*

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A unique mannose-binding lectin, highly specific for terminal Man(α1,3)Man groups, was isolated from bulbs of crocus (*Crocus vernus* All.). The lectin failed to bind to a mannose affinity column and was purified by simple gel permeation chromatography (Sephacryl S200). The purified lectin, obtained in crystalline form, had a molecular mass of 44 kDa on gel filtration and showed a single peptide band with a molecular mass of 11 kDa on SDS-polyacrylamide gel electrophoresis, indicating it to be a tetrameric protein composed of four identical subunits. The N-terminal amino acid sequence analysis of the crocus lectin showed essentially no homology with that of other mannose-binding bulb lectins. The crocus lectin selectively interacted with the wild type *Saccharomyces cerevisiae* and other mannans carrying terminal Man(α1,3)Man but not with those lacking this disaccharide unit. In hapten inhibition studies, methyl α-mannopyranoside did not inhibit the mannan-lectin interaction. Of various α-mannooligosaccharides, those having the Man(α1,3)Man sequence showed the highest inhibitory potency, confirming the strict requirement of lectin for terminal α1,3-linked mannosylmannose units. An affinity column of immobilized lectin enabled the complete resolution of yeast mannan and glycogen. The immobilized lectin may provide a useful tool for purification and analysis of biologically important polysaccharides and glycoproteins.

Since the first report on a yeast mannan-binding lectin from bulbs of tulip, *Tulipa generiana* (1), several kinds of α-mannose-binding lectins have been studied in our laboratory, mostly from bulbs of the family *Amaryllidaceae*, such as *Galanthus nivalis* (snow drop; GNA)[1] (2, 3), *Hippeastrum hybrid* (amaryllis), *Narcissus pseudonarcissus* (daffodil) (4), *Sternbergia lutea* (5), and *Allium sativum* (garlic) (6), which belongs to the *Lilaceae* family. A similar lectin was also isolated from leaves of *Listera ovata* (twayblade) (7). These lectins are distinct from hitherto known mannose/glucose-binding lectins, such as concanavalin A and other legume lectins, in their strict requirement for the axial C-2 hydroxyl group of α-D-mannopyranose. Our detailed studies of the carbohydrate binding specificity of these lectins have indicated some differences with regard to the location of mannosidic linkages at the terminal and/or internal position in the carbohydrate chain. For instance, GNA recognizes terminal Man(α1,3)Man (3) and also certain internal linkages (8). Similarly, *L. ovata* lectin can recognize the internal sequence of α(1,3)-linked mannosidic linkages (7). In a survey of new plant lectins we found that the bulbs of *Crocus vernus* All., belonging to the family *Iridaceae*, accumulates a very unique mannose-binding lectin with a very strict requirement for *terminal* α-1,3-mannosyl mannose units. This lectin, designated CVA, agglutinates rabbit but not human erythrocytes and does not appear to have homology with hitherto known mannose-binding lectins in its sequence of N-terminal amino acids.

This paper reports the purification, characterization, and detailed binding specificity of the crocus lectin, as revealed by interactions with a series of structurally defined yeast mannans, haptenic inhibition studies using a series of synthetic branched manno-trisaccharides, substituted at O-3 or O-6 of the α-mannose units. The application of the immobilized lectin for the selective fractionation of mannans and plant glycoproteins is also described.

EXPERIMENTAL PROCEDURES

Isolation of CVA—CVA was isolated from the crude extract of bulbs of spring flowering crocus. The peeled bulbs of *C. vernus* All. (160 g, water content 72%), cultivated in Niigata prefecture, Japan, in October 1994, were homogenized with 10 mM phosphate-buffered saline (PBS, pH 6.8) containing 0.1 M $(NH_4)_2SO_4$ solution overnight at 10 °C, and the PBS extract was centrifuged. To the supernatant of the extract was added $(NH_4)_2SO_4$ to 30% saturation, and the precipitate was collected, dialyzed against distilled water, and lyophilized (220 mg). Because CVA was not retained on a mannose-agarose (Sigma) column normally used for the isolation of most mannose-binding lectins, subsequent purification was carried out by gel filtration chromatography. The crude lectin (100 mg) obtained by $(NH_4)_2SO_4$ precipitation was dissolved in a minimum volume of PBS and applied onto a Sephacryl S200 (Pharmacia Biotech Inc.) column (2.5 × 200 cm) equilibrated with PBS; elution was conducted with the same buffer. The protein peak(s) was monitored by absorption at 280 nm, and the lectin activity was tested by a precipitation reaction with *Saccharomyces cerevisiae* mannan. The lectin-containing fractions were collected and dialyzed against distilled water, concentrated, and lyophilized (yield, 65 mg).

CVA Affinity Column—To purified CVA (15 mg) dissolved in 100 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl (2 ml) was added 700 mg of AF-Tresyl Toyopearl 650 (Toso Co.) in 5 ml of the same buffer. The mixture was shaken gently for 6 h at 25 °C and kept for a further 4 h at 10 °C according to the technical specification. The reaction product was filtered and washed with 100 mM phosphate buffer (pH 6.8) in 0.5 M NaCl, and the free tresyl-groups were blocked with 100 mM Tris-HCl buffer (pH 8.0) for 1 h at 25 °C. Determination of protein in the washing solution indicated approximately 70% of the lectin was conjugated to the Toyopearls.

For affinity chromatographic resolution of mannans and other polysaccharides or glycoproteins, each mannan or glycoprotein (1 mg) was applied to the CVA-toyopearl column (0.5 × 5 cm) and eluted at 5 °C, first with PBS, and then with 20 mM diaminopropane (DAP). Elution was monitored by measuring absorption at 280 nm (protein) or determination of mannose at 490 nm by the phenol-sulfuric acid method (9)

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[1] The abbreviations used are: GNA, snowdrop *G. nivalis* lectin; CVA, *C. vernus* lectin; DAP, diaminopropane; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatograph(y).

in a microscale system consisting of 100 μl of sample solution, 150 μl of 5% phenol, and 750 μl of concentrated H_2SO_4. When necessary, the glycan (glycoconjugate) eluted with DAP was collected, immediately dialyzed against water, and lyophilized.

Polysaccharides and Glycoproteins—The mannan of wild type *S. cerevisiae*, designated normal yeast mannan, was purified in our laboratory from the cell walls of bakers' yeast, as was sake yeast Kyoukai number 7. This mannan, isolated from the mechanically disintegrated cell walls, was further purified by digestion with glucoamylase to remove contaminating glycogen (10). Among various structurally different yeast mannans, the mannan of 4488 strain (mn 1 mutant) lacks α-1,3-mannosyl linkages (11), the mannan of *Candida albicans* NIH B-792 strain contains both terminal and internal αMan(1,3)Man linkages, and the *Candida parapsilosis* IFO 1396 strain lacks terminal but contains internal α-1,3-linkages (12). α-1,2-Mannosidase-treated mannan was a gift from Dr. T. Nakajima, Tohoku University.

Elsinan, a exocellular glucan of *Elsinoe leucospila*, was available from a previous study (13). Dextran B-1355-S was a gift of Dr. A. Jeans (Peoria, IL). Lima bean lectin and *Phaseolus vulgaris* lectin were available in our laboratory.

Oligosaccharides—All the monosacharides and their methyl or *p*-nitrophenyl glycosides were purchased commercially or were available in our laboratories. Man(α1,3)Man, Man(α1,3)Man-α-OMe, and Man(α1,6)Man-α-OMe were purchased from Sigma. Man(α1,2)Man-α-OMe, Man(α1,6)[Man(α1,3)]Man-α-OMe, and Man(α1,6)[Man(α1,3)]-Man(α1,6)[Man(α1,3)]Man-α-OMe were purchased from Pfanstiel Laboratories Inc. Man(α1,3)[Gal(α1,6)]Man-α-OMe, Man(α1,3)[Glc(α1,6)]-Man-α-OMe, and Man(α1,6)[Glc(α1,3)]Man-α-OMe Man(α1,6)[Gal-(α1,3)]Man-α-OMe were available at our laboratory (University of Michigan). Gal(α1,3)ManOMe (14) and Manα(1,3)Glc (15) were synthesized in our laboratory.

Molecular Mass Determination—The molecular mass of the purified CVA was estimated by gel filtration chromatography using Sephacryl S200 column (1.5 × 120 cm) equilibrated with 100 mM PBS (pH 6.8). The column was calibrated with thyroglobulin (670 kDa), gamma globulin (158 kDa), bovine serum albumin (66.2 kDa), ovalbumin (44 kDa), myoglobin (17.5 kDa), and cobalamin (1.3 kDa); CVA (1.2 mg) was applied to the column and eluted with the same buffer, each 1.0-ml fraction being assayed for protein by determining absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis (PAGE)—PAGE was conducted with an ATTO Mini-Slab electrophoresis, model AE-6000, using a precast 15% sulfate-polyacrylamide gel (SPU-15S) for molecular range, 1–6 × 10^6, or 12.5% sulfate-polyacrylamide gel (NPU-15L; 1.4–8 × 10^4), in the presence (reduced PAGE) or the absence of 2-mercaptoethanol (native PAGE). Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Molecular mass markers were low range protein markers (Promega) containing carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.4 kDa), myoglobin (16.9 kDa), lysozyme (14.4 kDa), and CNBr-cleaved myoglobin (8.1, 6.2, and 2.5 kDa), and also a molecular standard protein kit (Pharmacia).

Periodate Modification of Mannan and Oligosaccharide—*S. cerevisiae* mannan (10 mg in 1 ml) was oxidized with 50 mM sodium periodate at 10 °C for 5 days. After decomposition of the excess periodate with ethylene glycol, the oxidized mannan was reduced with sodium borohydride at 25 °C for 3 h, the excess borohydride was decomposed by stirring with Amberlite IR 120 (H^+-form), the product was dialyzed, and the oxidized-reduced mannan in the nondialyzable was lyophilized. An aliquot of Man(α1–3)ManOMe was also subjected to periodate oxidation and reduction, and the product was purified by passage through an Econo-pack 10 DG (Bio-Rad).

Quantitative Precipitation and Hapten Inhibition—Quantitative precipitation reactions were conducted essentially by the method of So and Goldstein (16). Varying amounts of polysaccharides or glycoproteins in microcentrifugal tubes were interacted with 20–25 μg of CVA, each in a total volume of 150 μl of 50 mM PBS (pH 6.8). After incubation at 35 °C for 1 h, the reaction mixture was kept at 5 °C for 48 h, centrifuged, and analyzed for protein in the precipitate by the micro Lowry method (17). For hapten inhibition studies, varying amounts of haptenic saccharides were added to the reaction mixture containing 20 μg of CVA and 20 μg of *S. cerevisiae* native mannan in a total volume of 150 μl. Protein in the precipitates of the reaction mixtures was determined using bovine serum albumin as standard, and the inhibition ratios were calculated.

pH Profile of Precipitation Reaction—A point on the yeast mannan (*S. cerevisiae*)-CVA precipitation curve was selected, and the amount of protein precipitated was determined at several pH values. Present in the reaction mixture were CVA (27 μg) and mannan (22 μg) in a total of 150 μl of 50 mM buffer of the following composition: glycine-HCl (pH 2.0–3.5); acetate (pH 4.0–5.5); phosphate (pH 6.0–8.3), and glycine-NaOH (pH 8.5–10.4).

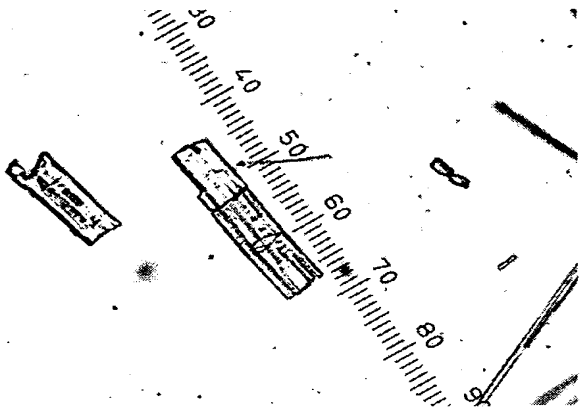


FIG. 1. **Crystalline lectin from *C. vernus* bulbs.**

Amino Acid Analysis—The purified lectin (40 μg) transferred onto polyvinylidene difluoride membrane was hydrolyzed with 6 M HCl for 21 h at 110 °C. Amino acid analysis was performed with a Shimazu high performance liquid chromatograph (HPLC) LC-10A.

N-terminal Amino Acid Sequence Analysis—Amino acid sequencing analysis was carried out on an ABI 4473 (automated sequencer) at the Protein Structure Facility of the University of Michigan Medical School. The subunit (11 kDa) of CVA on SDS-PAGE was transferred onto a polyvinylidene difluoride membrane and was digested with Asp-N or Lys-C for 18 h at 37 °C. The endoproteinase digestion products were subjected to reverse phase HPLC, elution being monitored by $A_{220\ nm}$. Amino acid sequence analysis of the peptide fractions was carried out as described above.

RESULTS

Purification of CVA—A preliminary study showed that the PBS extract of C. vernus bulbs, which agglutinated rabbit erythrocytes, interacted with yeast mannan but not with α-glucans, *e.g.* glycogen, dextran, etc., suggesting that it contained an α-mannose-specific lectin. The lectin in the crude protein extract was precipitated with 30% saturated $(NH_4)_2SO_4$. Interestingly, however, the lectin was not retained on a mannose column that had been used for the isolation of most α-mannose-binding lectins. Therefore, the crude lectin fraction obtained by $(NH_4)_2SO_4$ precipitation was applied to a Sephacryl S200 column (2.5 × 200 cm). Elution with 10 mM PBS indicated the presence of a single, symmetrical protein peak that interacted with *S. cerevisiae* mannan. It was rechromatographed on the same column, concentrated, dialyzed against distilled water, and lyophilized. Thus, this simple gel filtration procedure afforded the lectin (CVA) in a pure state; for example, 100 mg of the crude lectin extract yielded 67 mg of pure lectin.

The above purified CVA crystallized spontaneously in the form of needles or pillars when its concentrated aqueous solution (approximately 10 mg in 200 μl of water) was stored at 5 °C for several weeks (Fig. 1).

Purified CVA gave a molecular mass of 44,000 Da as estimated by gel filtration on a Sephacryl S200 column, which was calibrated with standard proteins (Fig. 2). Upon SDS-PAGE, the purified lectin gave a single polypeptide band of 11,000–11,500 Da both in the absence and the presence of 2% β-mercaptoethanol. Figs. 2 and 3 show the gel filtration profile and the results of SDS-polyacryamide gel electrophoresis. These results indicate CVA to consist of four identical subunits with no intersubunit disulfide bonds.

Chemical Characteristics of CVA—As noted above, the purified CVA, obtained as crystals, is a protein composed of four

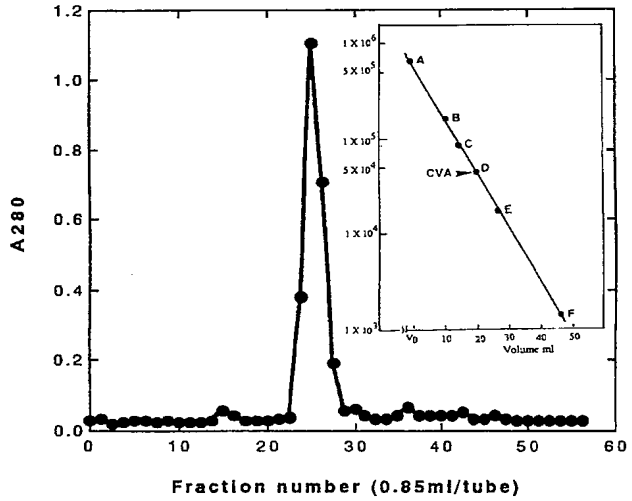


FIG. 2. **Gel filtration of CVA on Sephacryl S200.** The column (1.5 × 120 cm) was loaded with 2 mg of the purified lectin and eluted with 0.05 M PBS (pH 6.8). The molecular mass standards were thyroglobulin (670 kDa) (*A*); gamma globulin (158 kDa) (*B*); bovine serum albumin (66.2 kDa) (*C*); ovalbumin (45 kDa) (*D*); myoglobin (17 kDa) (*E*); and vitamin B_{12} (*F*). Fractions of 0.85 ml were collected, and proteins were assayed by absorbance at 280 nm.

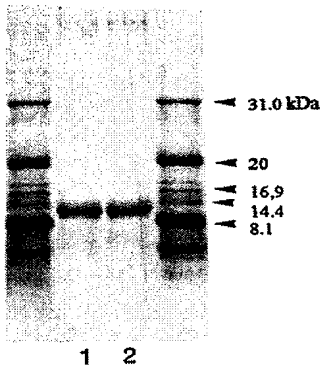


FIG. 3. **SDS-polyacrylamide gel electrophoresis of purified CVA.** *Left* and *right lanes*, standards: carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa); myoglobulin (16.9 kDa); lysozyme (14.4 kDa); and CNBr-cleaved myoglobin (8.1–6.2 kDa). *Lane 1*, native CVA. *Lane 2*, CVA in the presence of β-mercaptoethanol.

subunits of a single polypeptide with a molecular mass of 11,000 Da; it is devoid of carbohydrate, similar to other mannose-binding lectins of *Amaryllidaceae* bulbs. CVA contains essentially no metals (metal analysis showed only 0.04% of metal cations, thought to be Ca^{+2}).

The amino acid composition of CVA appeared somewhat similar to that of GNA, the mannose-binding lectins of the *Amaryllidaceae* family, as shown in Table I. The CVA molecule was estimated to consist of 410 amino acid residues. The lectin contains high proportions of asparagine/aspartic acid, glycine and leucine, like those found in GNA (2). It is also interesting that CVA contains a small proportion of methionine that is absent in GNA. The molecular mass (44,071–44,139 Da, calculated from the amino acid data) was consistent with the apparent molecular mass of 44,000 Da obtained by gel permeation chromatography. Therefore, assuming that the subunit mass is 11 kDa, the CVA molecule must contain four identical single peptide chains, each consisting of 102 amino acid residues.

The N-terminal amino acid sequencing analysis of CVA provided a partial N-terminal amino acid sequence. Table II indicates that the molecular structure of CVA is strikingly different from the hitherto characterized mannose-binding lectins of *Amaryllidaceae*; the N-terminal sequence of 24 amino acids of CVA indicated that its homology with GNA is only 8.3%, whereas the *S. lutea* lectin possesses 76% homology with GNA (7).

TABLE I
Comparison in amino acid compositions of lectins of CVA and GNA

Amino acid	CVA		GNA
	mol %	*Residues/subunit*	*mol %*
Asx	22.0	22	15.4
Thr	5.9	6	7.5
Ser	7.3	7	10.5
Glx	8.5	9	7.2
Pro	3.5	4	4.1
Gly	9.0	9	12.0
Ala	6.2	6	3.5
Cys	2.0	3	1.7
Ile	3.2	3	5.4
Leu	7.6	8	8.9
Tyr	2.8	3	5.0
Phe	2.9	3	2.1
His	2.8	3	1.0
Lys	2.3	3	4.2
Arg	4.5	4	3.5
Val	7.1	7	4.8
Met	2.4	3	0.0
		(Total 102)	
Molecular mass	44,000		50,000
Subunit mass	11 kDa		12 kDa

TABLE II
Comparison of partial N-terminal amino acid sequence of CVA and two Amaryllidaceae lectins, GNA and SLA

	1 5 10 15 20 25
CVA	NIPQVRNVLFSSQVMYDNAQLATR
	1 5 10 15 20 25
GNA	DNILYSGETLSTGEFLNYGSFVFVFIM
	1 5 10 15 20 25
SLA[a]	DNYLYSGETLFSGQFLNYGNYRFIM

[a] SLA, S. lutea lectin.

Interactions of CVA with Polysaccharides and Glycoproteins—Preliminary precipitation studies showed that CVA interacts to form a precipitate with the mannan of ordinary yeast (*S. cerevisiae*) and some plant glycoproteins, such as lima bean lectin (18) and *P. vulgaris* lectin (19), but not with α-D-glucans (glycogen, dextran, etc.), ovalbumin, and ovomucoid. To ascertain the mannosyl linkage specificity of CVA, structurally different yeast mannans whose repeating unit structures have been well established (Fig. 4, *I–V*) were examined by quantitative precipitation. As shown in Fig. 5. CVA strongly precipitated normal types of yeast mannans, *i.e.*, bakers' yeast (*S. cerevisiae*) and *S. cerevisiae* Kyoukai number 7 mannan, both having essentially the same branched structure (Fig. 4, *I*), but dextran 1355S, having numerous α1,3-glucosidic linkages (20), did not react. Elsinan, a fungal linear α-1,3/1,4 glucan (15) reacted only slightly, as in the case of GNA (3), indicating that CVA specifically recognizes the α-mannosyl configuration. Interestingly, CVA does not interact with mannans that are devoid of Man(α1,3)Man units at their nonreducing termini (Fig. 4). Fig. 6 shows precipitation curves of CVA with these structurally different mannans, *i.e.*, the normal yeast mannan of *S. cerevisiae* and other mannans, including enzymatically α-1,2-mannose-deleted *S. cerevisiae* mannan (Fig. 4, *IV*), *C. albicans* B-792 mannan, which contains branched α-1,3-mannosyl termini (Fig. 4, *V*), the α-1,3-mannose-deficient mannan of *S. cerevisiae* 4484 mutant strain (Fig. 4, *III*), and *C. parapsilosis* 1396 mannan (Fig. 4, *II*) containing internal (1,3)-linked

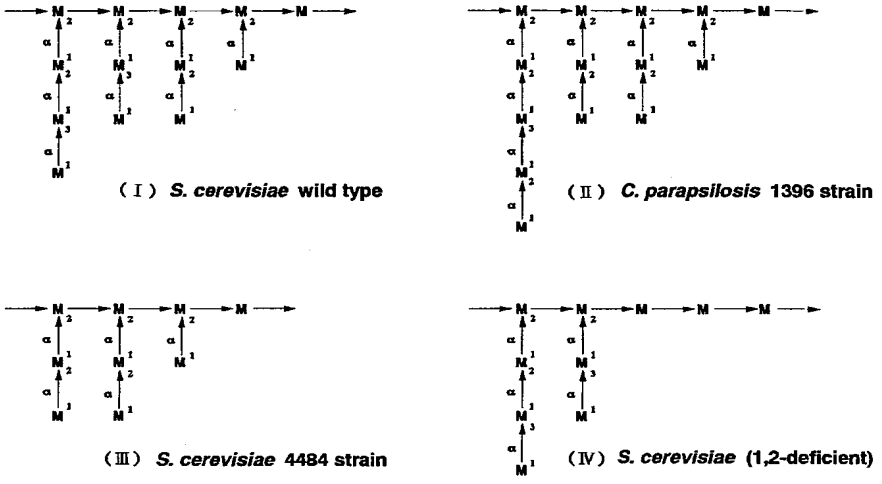
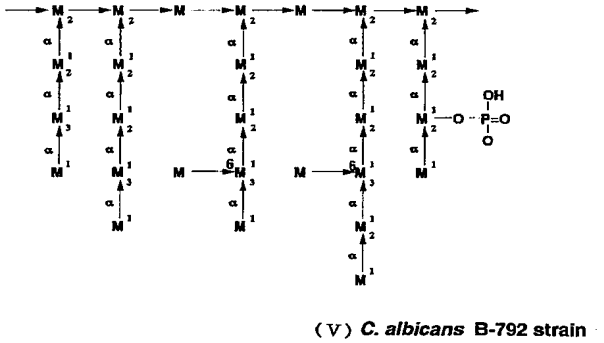


FIG. 4. **Repeating unit structure of various yeast mannans used for interaction with CVA.**



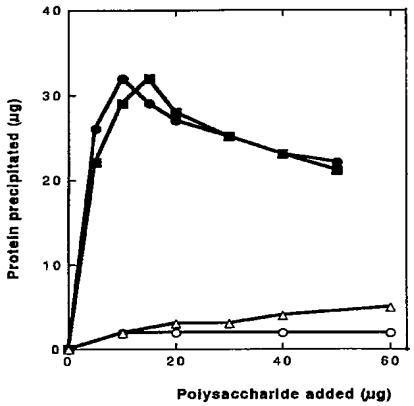


FIG. 5. **Quantitative precipitation curves between CVA and "normal" yeast mannans (see Fig. 4) and α-D-glucans.** Shown are mannan of *S. cerevisiae* (bakers' yeast, ●); ■ *S. cerevisiae sake* (Sake yeast, ■); *Leuconostoc mesenteroides* 1355S dextran (○); and Elsinan of *E. leucospila* (△). Varying amounts of polysaccharides were incubated with 35 μg of CVA in a volume of 150 μl, and the amount of protein precipitated in each tube was determined by the Lowry method (17).

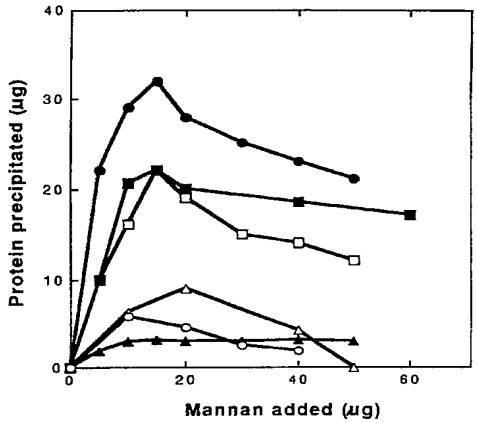


FIG. 6. **Quantitative precipitation curves of CVA and structurally different yeast mannans (see Fig. 4, *I–V*).** ●, *S. cerevisiae* (*I*); ○, $NaIO_4$ and $NaBH_4$-treated *I*; △, *C. parapapslosis* 1936 (*II*); ▲, *S. cerevisiae* 4484 mutant (*III*); ■, enzymically generated terminal (α1,2)Man-deleted *S. cerevisiae* mannan (*IV*); □, *C. albican* B-792 manan (*V*).

mannose residues not adjacent to their termini. Among these mannans it is striking that mannan *III* (Fig. 4), deficient in terminal α-Man(1,3)Man, did not interact with CVA. On the contrary, under the same conditions, GNA and the *S. lutea* lectin gave appreciable precipitation with III (data not shown). The recognition of α-Man(1,3)Man by CVA is confined to the terminal position, because *C. parapsilosis* 1396 mannan (Fig. 4, *II*), which contains internal residues, did not react. The periodate-oxidized and borohydride-reduced *S. cerevisiae* mannan was no longer reactive with CVA, even though the intact α-(1,3)-mannose units are present adjacent to the modified end groups (Fig. 6). These quantitative precipitation reactions con-

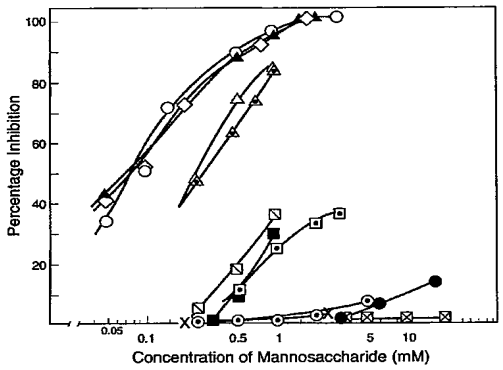


FIG. 7. **Hapten inhibition of the CVA *S. cerevisiae* mannan precipitation system (see text and Table III).** ○, Man(α1,3)Man-α-OMe; ▲, Man[(α1,6)[Man(α1,3)]Man-αOMe; ◇, Man(α1,6)[Man(α1,3)]-Man(α1,6) [Man(α1,3)]Man-α-OMe; △, Man(α1,3)[Glc(α1,6)]Man-α-OMe; ◬, Man(α1,3)[Gal(α1,6)] Man-α-OMe; ◱, Man(α1,6)[Gal(α1,3)]Man-α-OMe; ■, Man(α1,6)[Glc(α1,3)]Man-α-OMe; ⊡, Man(α1,6)Man-α-OMe; ⊙, Man(α1,3)Glc; ×, Gal(α1,3)ManαOMe; ●, Man(α1,2)Man-α-OMe; ⊠, ManαOMe.

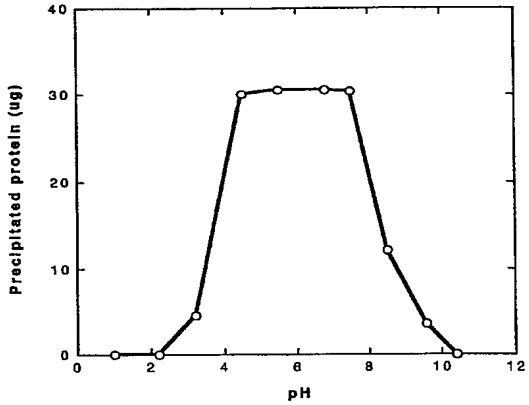


FIG. 8. **Effect of pH on the precipitation reaction between CVA and *S. cerevisiae* mannan.** Each microcentrifuge tube contained CVA (27 μg) and mannan (22 μg) in a total volume of 150 μl of 50 mM buffer. See "Experimental Procedures" for buffer composition.

firmed that CVA possesses a unique, highly specific binding specificity with regard to its α-1,3-mannosyl linkages and their localization. Due to its highly selective binding property, CVA was not retained on the mannose column, as described in the purification procedure.

Inhibition of Precipitation Reaction by Haptenic Mannosaccharides—To confirm the binding specificity of CVA, detailed inhibition studies were conducted using the precipitation system of CVA with the *S. cerevisiae* mannan under maximum precipitating conditions. Mannose, glucose, and other monosaccharides showed no inhibition. Methyl α-mannopyranoside, known as one of the best inhibitors for most mannose-specific lectins, was found to be a very poor inhibitor; it does not inhibit up to 30 mM, and at 100 mM gave only 20% inhibition. This result is consistent with the fact that CVA does not bind to single α-mannosyl end groups of any branched mannan. Inhibition of a series of linear and branched mannosaccharides were compared. The inhibition curves of these oligosaccharides are depicted in Fig. 7 and in Table II, in which the percentage of inhibition of the oligosaccharides at various concentrations are compared. Some oligosaccharides exhibited very poor inhibition and are not presented at 50% inhibition concentrations. According to the inhibition curves of mannooligosaccharides, they may be divided into several groups (Fig. 7). The best inhibitor group includes those having the Man(α1,3)Man sequence, such as, Man(α1,3)Man-α-OMe, the branched trisaccharides, *i.e.*, Man(α1,6)[Man(α1,3)]Man-α-OMe, and Man-(α1,6)[Man(α1,3)]Man(α1,6) [Man(α1,3)]Man-α-OMe. These all gave very similar inhibition curves with 50% inhibition at approximately 0.07 mM concentrations. The second best inhibitors are branched trisaccharides, *i.e.* Man(α1,3)[Glc(α1,6)] Man-α-OMe and Man(α1,3)[Gal(α1,6)]Man-α-OMe, both also containing the sequence of Man(α1,3)Man. They gave 50% inhibition at 0.3 mM. The oligosaccharides belonging to the third group exhibited 30% inhibition at approximately 1 mM; they include Man(α1,6)Man-α-OMe and those having α(1,3)glucosyl or α(1,3) galactosyl branches, *i.e.* Man(α1,6)[Glc(α1,3)]Man-α-OMe and Man(α1,6)[Gal(α1,3)]Man-α-OMe. Man(α1,2)Man-α-IMe was much less active. It must be noted that both Man(α1,3)Glc and Gal(α1,3)ManαOMe are not active inhibitors, indicating that binding to CVA must involve recognition of the terminal Man(α1,3)Man disaccharide unit. In connection with these findings, when Man(α1,3)Man-α-OMe, a most potent inhibitor, was periodate-oxidized and reduced, the resulting terminally modified α-1,3-mannosaccharide was no longer active, supporting this unique binding specificity of CVA.

The pH Profile of the Manan S. cerevisiae CVA Precipitation Reaction—The pH profile of the precipitation reaction from pH 2 to 10 is depicted in Fig. 8. A plateau of constant protein precipitation was demonstrated over the pH range 4.5–7.5.

Binding Characteristics of CVA Affinity Column—An aliquot of CVA was conjugated to AF-tresyl Toyopearl 650 (binding efficiency, 70%), and its binding capacity for polysaccharides and plant glycoproteins carrying α-1,3-mannosyl ends was investigated. As anticipated, normal yeast mannan of *S. cerevisiae* bound strongly to the CVA column and was eluted by weak alkali, such as 20 mM DAP, whereas the mannan of the 4484 mutant was not bound (Fig. 9, *A* and *B*). *C. parapsilosis* 1936 mannan also was not bound. As glycogen passed through the column unretarded, complete resolution of the mannan and glycogen in the yeast cell can be made (Fig. 9*C*), as previously reported for the *L. ovata* lectin affinity column (7). Some glycoproteins are known to contain high mannose-type carbohydrate chains. Among them are lima bean lectin (*P. lunatus*) and Tora bean lectin (*P. vulgaris*), both of which contain α-1,3-mannosyl end group(s) in their glycan structures (18, 19). As shown in Fig. 10, they could also bind to the CVA affinity column and were readily eluted with dilute DAP.

DISCUSSION

C. vernus, the spring flowering crocus, belongs to the family *Iridaceae*. It originated in middle Europe and was brought to Japan one hundred years ago. We found that its PBS extract precipitated bakers' yeast mannan but did not strongly agglutinate yeast cells, compared with concanavalin A and GNA. Furthermore, unlike ordinary glucose/mannose and other mannose-binding lectins, this lectin (CVA) did not bind to a mannose affinity column. Nevertheless, it was purified by successive $(NH_4)_2SO_4$ precipitation and gel chromatography (yield, 145 mg/160 g bulbs).

The purified lectin, which crystallized spontaneously, is a carbohydrate-free protein (mass = 44,000 Da) composed of four identical peptide chains of about 100 amino acid residues; there was no evidence of disulfide interchain bonds. It was somewhat surprising that its N-terminal amino acid sequence of 24 residues showed essentially no homology with GNA and other *Amaryllidaceae* mannose-specific lectins (Table II), although its molecular size and amino acid composition are very similar to those of GNA and other bulb lectins (Table I). These results strongly suggest that the molecular structure of the CVA car-

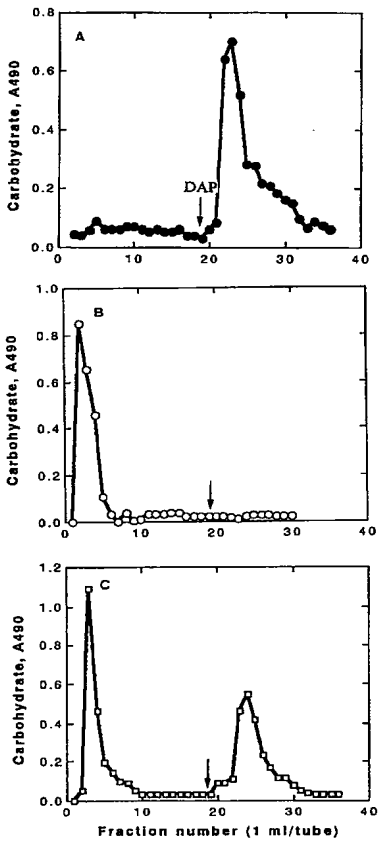


FIG. 9. **Elution profile of *S. cerevisiae* mannan (*A*), *C. parapsilosis* 1396 mannan (*B*), and mannan and glycogen (1:1 mixture) of *S. cerevisiae* (*C*).** Each polysaccharide (1 mg) was applied to CVA-Toyopearl column (5 ml). After elution with 0.05 M PBS, the polysaccharide retained on the column was eluted with 20 mM DAP, as indicated by the *arrows*. Carbohydrate was monitored by the phenol-sulfuric acid method at 490 nm.

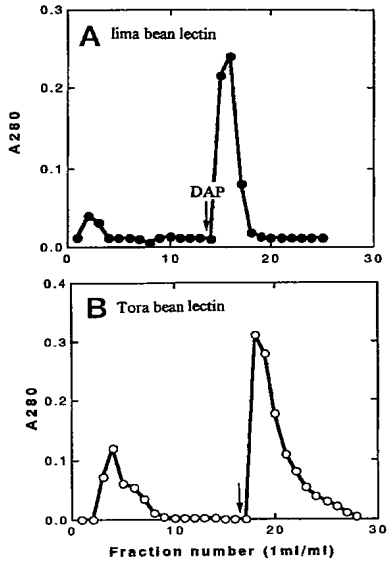


FIG. 10. **Elution profile of some plant glycoproteins by CVA-Toyopearl column.** *A*, lima bean lectin. *B*, Tora bean (*P. vulgaris*) lectin. Protein was assayed at 280 nm.

TABLE III
Inhibition by mannosaccharides of S. cerevisiae mannan precipitation with CVA

Mannosaccharide	Concentration	Inhibition
	mM	%
ManαOMe	30	0
	40	7.4
	100	21.5
p-NP-αMan	6	2.5
	15	5.0
Man(α1,3)ManαOMe	0.05	32.7
	0.1	50.9
	0.2	72.2
	1.6	98.0
Oxidized, reduced-Man(α1,3)ManαOMe	1.0	10.0
Man(α1,6)Man-α-OMe	2.0	33.0
	3.0	36.4
Man(α1,2)ManαOMe	6.0	8.5
	16.0	16.6
Man(α1,3)Glc	2.0	2.0
	4.0	7.5
Gal(α1,3)ManαOMe	(No inhibition at 10 mM)	
αMan_6 ↓ Man(α1,3) ManαOMe	0.1	52.5
	0.5	79.9
	1.0	87.2
αGlc_6 ↓ Man(α1,3) ManαOMe	0.5	74.5
	1.0	83.5
αGal_6 ↓ Man(α1,3)ManαOMe	0.5	63.6
	1.0	85.3
αMan_6 Man_3 ↓ ↓ Man(α1,3)Man(1,6)ManαOMe	0.1	49.0

bohydrate-binding sites must be different from *Amaryllidaceae* bulb lectins.

The precipitation reactions of CVA with various types of yeast cell wall mannans showed that this lectin possesses an unusual, very strict binding specificity. It reacted only with the mannans having terminal α-1,3-mannosyl groups, such as normal yeast mannan (Fig. 4, *I*), the mannan lacking α-1,2-linked terminal mannose units (Fig. 4, *IV*), and *C. albicans* B-792 mannan (Fig. 4, *V*). However, it did not react with other types of mannans, such as those lacking α-1,3-linkage at its terminal ends or localized at internal positions, as in the case of the 4484 mutant mannan and of *C. parapilosis* 1396 (Fig. 4, *III* and *II*); these mannans contain nonreducing, terminal α-1,2-linked mannose residues.

The pH profile of the yeast mannan (*S. cerevisiae*) precipitation reaction indicates that the reaction is maximum over the pH range 4.5–7.5. It is possible that acidic residue(s) are involved in the carbohydrate-binding site of CVA inasmuch as the ascending portion of the pH profile (pK = ~3.5–4.0) is in the titration range of β- and γ-carboxyl groups of Asp and Glu. Acidic amino acid residues have been identified in many lectin-binding sites including the snowdrop lectin (22).

Detailed inhibition studies were conducted using the normal *S. cerevisiae* mannan-CVA precipitation system with various mannobioses and branched tri- and pentasaccharides containing α-1,3- and/or α-1,6-terminal mannosyl group(s). The results clearly indicate that only those having α-1,3-mannosidic termini, either linear or branched, exhibited strong inhibitory activity. Thus, Man(α1,3)Man-α-OMe, Man(α1,3)[Man(α1,6)]-Man-α-OMe, and Man(α1,6)[Man(α1,3)]Man(α1,6) [Man(α1,3)]-Man-α-OMe were the best inhibitors. When the nonreducing

terminal mannose group is periodate-modified, its original activity is destroyed. This group of oligosaccharides was 4.5 times more active than α-1,3 mannobiose substituted at the O-6 position of the "reducing" mannosyl group with a glycosyl group other than an α-mannosyl group, *e.g.* Man(α1,3)[Glc(α1,6)]-Man-α-OMe and Man(α1,3)[Gal(α1,6)]Man-α-OMe. These results suggests that a glycosyl substituent at the O-6 position of the reducing α-mannosyl residue is tolerated by the CVA-binding sites. The α-1,6-linked mannosylmannose unit, with or without an *O*-3-glucosyl or galactosyl branch were much less active. Man(α1,3)Glc and Gal(α1,3)ManαOMe were very poor inhibitors, slightly more active than methyl α-mannoside, which showed no inhibition at a concentration of 30 mM (Table III). Thus, these hapten inhibition studies clearly confirm that terminal α1,3-linked mannosyl mannose units are required for binding to the lectin.

Our previous study indicated that GNA of the *Amaryllidaceae* family, also has a high affinity for terminal Man(α1,3)-Man units (3), but the binding specificity of GNA appears more broad than CVA, because GNA is also able to recognize internal (1,3/1,6) mannose units (8, 21), and it precipitated periodate-modified *S. cerevisiae* mannan as well as the 4484 mutant mannan (data not shown).

Recently the three-dimensional structure of GNA was elucidated and its binding site for α-methyl mannoside in each monomer was identified (22). With regard to molecular structure, CVA also is a tetrameric protein with similar molecular size and amino acid composition as that of GNA. CVA is rich in Asn, Leu, and Gly, like GNA, but the N-terminal amino acid sequence is completely different. Therefore, it will be of great interest to compare the three-dimensional structure of the two lectins. We have succeeded in crystallizing CVA, and its crystallographic x-ray structure is in progress.

The biological role of plant lectins is not fully understood and remains the subject of current debate, although their role as a host defender against animals, fungi, bacteria, or virus is an attractive hypothesis (23). Apart from such a biological role, the aforementioned unique binding specificity of CVA prompted us to prepare the corresponding immobilized lectin and to study its application to the affinity chromatographic analysis of polysaccharides and glycoproteins. The normal mannan of *S. cerevisiae* was strongly bound to the column, but α-glucans, *e.g.* glycogen, starch, etc., and also certain yeast mannans genetically deficient in terminal α-1,3-mannosyl groups readily passed through the column. Some plant glycoproteins, for instance, lima bean lectin and *P. vulgaris* lectin, which contain 5% carbohydrate chains carrying two α-1,3-mannosyl terminal groups, were retained on CVA column and were eluted with dilute DAP (Fig. 9); elution with methyl α-mannoside was unsuccessful. From these examples, it may be anticipated that immobilized CVA may provide a useful probe for selective fractionation of biologically important glycans and glycoproteins containing Man(α1,3)Man termini.

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Exhibit C

Reference 3

Eur. J. Biochem. 217, 677–681 (1993)

The α-mannosyl-binding lectin from leaves of the orchid twayblade (*Listera ovata*)

Application to separation of α-D-mannans from α-D-glucans

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The carbohydrate-binding specificity of an α-D-mannose-specific lectin isolated from leaves of the orchid twayblade (*Listera ovata*) was elucidated by quantitative precipitation of mannose-containing polysaccharides and glycoproteins, hapten inhibition, and affinity chromatography on the immobilized lectin. *L. ovata* agglutinin (LOA) interacted with various α-mannans and galactomannans of yeasts, fungi and bacteria, but not with α-glucans, e.g., dextran and glycogen, as do mannose/glucose-binding lectins. This lectin, LOA, appears to be highly specific for α1-3 mannosidic linkages. It reacted with a linear α1-3-mannan (D. P. 15) and, surprisingly, even with a linear α1-3-mannoheptasaccharide. The LOA/*C. tropicalis* mannan precipitation reaction was inhibited by α-linked mannooligosaccharides, in the order, α1-3 > α1-6 > α1-2 linkages; α1-3 $[Man]_4$ and $[Man]_5$ were the best inhibitors among various mannooligosaccharides tested, having 7-times greater potency than α1-3 $[Man]_2$, and 18-times that of methyl α-mannoside. LOA/mannan interaction was also inhibited by periodate-oxidized and reduced α1-3 $[Man]_5$ which had an inhibitory potency similar to that of α1-3 $[Man]_3$, confirming that LOA also recognizes the internal α1-3-mannosidic linkages of carbohydrate chains.

Complete resolution of mannan and glycogen from yeast cells, by affinity chromatography on an immobilized LOA column, and retention of several high-mannose-glycoproteins suggest this lectin to be a useful tool for purification and structural investigation of α-mannosyl-containing polysaccharides and glycoconjugates.

A series of α-D-mannosyl-specific lectins present in the bulbs of monocotyledonous plants has been reported by our laboratory [1–6]. The snowdrop (*Galanthus nivalis*, GNA), daffodil (*Narcissus pseudonarcissus*, NPA), amaryllis (*Hippeastrum hybr.*, HHA), garlic (*Allium sativum*, ASA), ramsons (*Allium ursinum*, AUA) and other related lectins are carbohydrate-binding proteins that are clearly different from the well-known mannose/glucose-binding lectins, such as concanavalin A and lectins from pea, lentil and *Vica faba* seeds [7], present in leguminous plants, in that they interact strongly with α-mannans and certain galactomannans of yeasts and fungi, but not with glycogen, amylopectin, dextran and other α-glucans.

The orchid twayblade (*L. ovata*) leaves accumulate a lectin (*L. ovata* agglutinin, LOA) which does not agglutinate human erythrocytes. LOA is a dimeric protein composed of two subunits of M_r 12500; it is the first lectin to be isolated from a species of the family Orchidaceae, and exhibits exclusive specificity towards D-mannose [8].

In this study, we report the carbohydrate-binding properties of the first lectin to be isolated from orchid leaves, as revealed by quantitative precipitation reactions with several microbial α-mannans and galactomannans, by hapten inhibition studies, and by affinity chromatography on the immobilized lectins.

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Abbreviations. ASA, *Allium sativum* agglutinin; AUA, *Allium ursinum* agglutinin; GNA, *Galanthus nivalis* agglutinin; HHA, *Hippeastrum* hybr. agglutinin; LBA, *Phaseolus lunatus* agglutinin; LOA, *Listera ovata* agglutinin; NPA, *Narcissus pseudonarcissus* agglutinin; PHA, *Phaseolus vulgaris* agglutinin; Me-α-D-Man*p*, methyl α-D-mannopyranoside.

MATERIALS AND METHODS

Purification of LOA

The LOA used in this study was purified from *L. ovata* leaves using a D-mannose column as reported previously [8].

Saccharides, polysaccharides and glycoproteins

Most monosaccharides and their methyl glycosides used in this study are available commercially. The α1-3-linked mannooligosaccharides and a low-molecular-mass α1-3-mannan (D. P. 15) were prepared by mild acid hydrolysis of the

glucuronoxylomannan of *Tremella fuciformis* [9]. Some α1-2-linked and α1-6-linked mannooligosaccharides were the gifts of Dr T. Nakajima of Tohoku University, Japan. Glc(α1-4) Man was available from previous studies. Man(α1-3) Man-α-*O*-Me was purchased from the Sigma Chemical Co. Man(α1-3)Man(α1-6) Man-α-*O*-Me was a gift from Dr G. Krepinsky, University of Toronto, Canada.

α-Mannans of *Saccharomyces cerevisiae* (Oriental Baker's yeast), *Candida tropicalis* [10], *Candida albicans*, *Aspergillus fumigatus* [11], *Alternaria kikuchiana* and *Mycobacterium tuberculosis* [12], and galactomannans of *Audiobasidium pullulans* [13] were prepared as described previously. *Candida lyptica* mannan (a gift of Dr Gorin), *Elucione leucospila* [14], and the arabinomannan of *M. tuberculosis* [12] were also available. The galactomannans of *A. fumigatus* and *A. kikuchiana* which contain galactofuranosyl side chains were treated with 0.1 M H_2SO_4 at 90°C for 1 h, and the resulting mannan preparations were used in precipitation studies. Dextran B-1355-S was a gift of Dr. A. Jeane, Peoria, IL. Ovalbumin was purchased from Sigma Chemical Co. Lima bean lectin (LBA) and *Phaseolus vulgaris* lectin (PHA) were available in our laboratories.

α1-3-linked mannan and mannooligosaccharides

Glucuronoxylomannan of *T. fuciformis* which contains an α1-3-linked mannan backbone was oxidized with 50 mM $NaIO_4$ for 7−10 days at 4°C, followed by reduction with sodium borohydride. The resulting polysaccharide polyalcohol was hydrolyzed with 0.4 M trifluoroacetic acid at 90°C for 5 h and a series of α1-3-linked mannooligosaccharides, including a linear α1-3 mannan (D. P. 15), was purified by gel filtration on a column of BIO-gel P-2; the content of 1-3-mannosidic linkages was confirmed by methylation analysis. An α1-3-mannopentaose was also subjected to periodate oxidation/reduction employing the same conditions, as described above.

Precipitation and hapten inhibition assay

Quantitative precipitation reactions were caried out by a microprecipitation technique [1]. LOA (20 μg or 50 μg) was added to varying amounts of polysaccharides in a total volume of 100, 150, 200 or 250 μl. After incubation at 30°C for 1 h the mixtures were kept at 4°C for 48 h and centrifuged, and protein in the precipitates was determined by the method of Lowry [15] using bovine serum albumin as a standard.

Sugar inhibition of the precipitation reactions was carried out by adding increasing amounts of sugar or derivative to precipitation systems containing LOA and *C. tropicalis* mannan.

Immobilization of lectin and affinity chromatography

An aliquot of purified LOA (10 mg) was immobilized by coupling with AF-Tresyl Toyopearl 650 (Toyosoda Co.). The LOA conjugated Toyopearl contained approximately 1.5 mg protein/ml gel.

Polysaccharides (250−1000 μg) or glycoproteins (100 μg carbohydrate) were applied to the LOA-Toyopearl column (1 cm×10 cm). The column was washed first with NaCl/P_i (10 mM sodium phosphate, 0.15 M NaCl, pH 7.2) followed by elution with NaCl/P_i containing 0.5 mM methyl α-D-mannoside (Me α-D-Man*p*). The amount of carbohydrate present in each tube was determined by the phenol/sulfuric acid method [16].

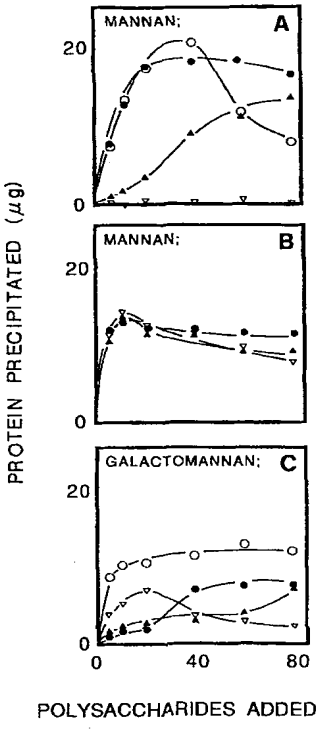

Fig. 1. Quantitative precipitation curves of yeast mannans (A, B), galactomannans and arabinomannan (C) bv LOA. The amount of protein was 50 μg (in 200 μl in each tube). (A) Mannans of *S. cerevisiae*,(●); *C. tropicalis*, (○); *A. fumigatus*, (▲); dextran 1355-S, (▽). (B) Mannans of *C. albicans*, (▽); *A. kikuchiana*, (●); *M. tuberkulosis Aoyama*, (▲); (C) Galactomannans of *A. pullulans*, (○); *C. lyptica*, (▽); *E. leucospila*, (●); and an arabinomannan, (▲) were also examined.

RESULTS AND DISCUSSION

Precipitation assay

The precipitation curves of LOA with various polysaccharides are shown in Fig. 1. LOA interacted strongly with the highly branched α-mannans isolated from *S. cerevisiae* and *C. tropicalis* [10] which contain side chains of α1-2-linked and α1-3-linked D-mannosyl residues attached to a backbone of α1-6-linked mannose residues. The lectin did not give a precipitation reaction with glycogen or dextran B-1355-S. The arabinomannan from *M. tuberculosis* Aoyama B [12], and cell surface galactomannans from *C. lyptica* and *E. leucospila* [14] in which some residues in the α1-6-mannose backbone, are substituted by α-D-galactofuranosyl and mannobiosyl or triosyl units gave approximately 25−50% the amount of protein precipitation as that with the *S. cerevisae* mannan. The differences in these precipitation reactions may be attributed to the structures of the mannans, particularly the position of short side chains of α1-3 or α1-2-mannose units, which may affect accessibility to the lectin. The essentially linear α1-3 mannan (D. P. 15) prepared from *T. fuciformis* glucuronoxylomannan [9] reacted strongly with LOA. This lectin also reacted with the linear α1-3-manno-

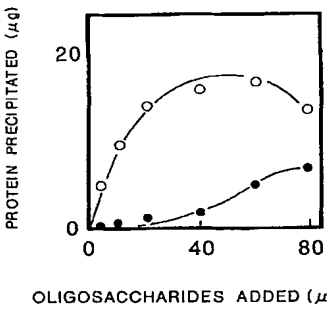

Fig. 2. Quantitative precipitation curve of a linear α1-3-mannan (D. P. 15) (○) and α1-3-Man$_7$ (●) by LOA. The protein concentration was 50 μg/tube.

Table 1. Inhibition by various sugars of LOA/*C. tropicalis* mannan precipitation.

Sugars	Concentration for 50% inhibition
	mM
D-Mannose	440
D-Glucose	No inhibition at 2000 mM
D-Galactose	No inhibition at 2000 mM
PNP α-D-Mannoside	32% inhibition at 67 mM
PNP β-D-Mannoside	12% inhibition at 80 mM
Me α-D-Mannoside	220
Me β-D-Mannoside	1000
Man (α1-2) Man	125
Man (α1-2) Man (α1-2) Man	23% inhibition at 50 mM
Man (α1-3) Man	85
Man (α1-3) Man (α1-3) Man	21
Man (α1-3) Man (α1-3) Man (α1-3) Man	14
Man (α1-3) Man (α1-3) Man (α1-3) Man (α1-3) Man	12
Periodate-oxidized, $NaBH_4$-reduced α1-3 $[Man]_5$	28
Man (α1-3) Man-α-*O*-Me	17
Man (α1-6) Man	100
Man (α1-6) Man (α1-6) Man	29% inhibition at 50 mM
Glc (α1-4) Man	14% inhibition at 50 mM
Man α1 \ 6 Man-α-*O*-Me 3 / Manα1	30

heptasaccharide to give a precipitation reaction as shown in Fig. 2. These results suggest that LOA recognizes sequences of internal α-D-mannosyl residues, in addition to terminal α-mannosyl units.

Inhibition of precipitation reaction by haptenic sugars

The carbohydrate-binding specificity of LOA was studied by sugar hapten inhibition of the interaction of LOA with *C. tropicalis* mannan. It was confirmed that LOA is a mannose-specific lectin and shown that neither D-glucose nor D-galactose inhibited the lectin/mannan reaction at a concentration of 2 M. Methyl α-D-mannopyranoside was twofold better an inhibitor than that of D-mannose (Table 1) whereas the

Table 2. Inhibition by manno-oligosaccharides of four α-mannosyl-binding lectins. Data for LOA are based on inhibition of the LOA/*C. tropicalis* mannan precipitation system. Data for *Galanthus nivalis* (GNA) bulb lectin are taken from Shibuya et al. [1]. Data for *Narcissus pseudonarcissus* (NPA) and *Hippeastrum hybr.* (HHA) bulb lectins are taken from Kaku et al. [3].

Sugar	Relative inhibitory potency on			
	LOA	GNA	NPA	HHA
D-Mannose	1.0	1.0	1.0	1.0
Me α-D-Mannoside	2.0	1.6	1.2	1.5
Man (α1-2) Man	3.5	2.1	3.3	3.2
Man (α1-3) Man	5.2	12.1	2.8	5.9
Man (α1-3) Man-α-*O*-Me	25.9	14.2	3.1	10.5
Man (α1-2) Man-α1-2 Man	> 8.8	3.4	1.7	3.6
Man (α1-3) Man (α1-3) Man	21.0			
Man (α1-6) Man (α1-6) Man	> 8.8	5.7	12.4	20.0
Man α1 \ 6 Man-α-*O*-Me 3 / Manα1	14.5	28.3	3.8	13.8

β-anomer (methyl β-D-mannopyranoside) was a poor inhibitor exhibiting only 25% the activity of the α-anomer.

Among a series of α-linked manno-oligosaccharides tested, it is apparent that the α1-3-mannopentasaccharide is the best inhibitor of the LOA/mannan interaction. As shown in Table 1 α1-3-mannobiose had a significantly higher inhibitory activity than α1-2 and α1-6 mannobiose indicating that the α1-3 linkage is most complementary to the sugar-binding sites of LOA. The α1-3 mannobiose had an inhibitory activity 2.5-times higher than that of the methyl α-D-mannoside. The mannotriose was 10-times, mannotetraose 15-times and mannopentaose 18-times more inhibitory than methyl α-D-mannoside. Man(α1-3)Man-α-*O*-Me was also an excellent inhibitor, better than Man(α1-3)Man, and nearly equivalent to Man(α1-3)Man(α1-3)Man, suggesting that the α-configuration of the reducing unit makes an important contribution to its binding activity. It is interesting to note that the inhibitory potency of the branched trisaccharide Manα1-3(Man-α-1-6)-Man-α-*O*-Me was approximately 50% that of Man(α1-3)-Man-α-*O*-Me suggesting it is the Man(α1-3)Man residue which is recognized by the lectin. The fact that periodate-oxidized and $NaBH_4$-reduced α1-3-mannopentasaccharide, in which both terminal mannosyl ends were modified, had the same activity as α1-3-linked mannotriose (Table 1), strongly suggests that the combining site(s) of LOA appears to be most complementary to three consecutive mannosyl units linked α1-3, even if both terminal mannosyl units are not involved. In similar fashion, LOA gave a strong precipitation with a linear α1-3-mannan (D. P. 15) and the periodate-oxidized and reduced glucuronoxylomannan of *T. fuciformis* hich contains an (α1-3)-mannan backbone. In Table 2, the mannosyl-binding specificity of LOA is compared with several other mannose-specific lectins.

Binding characteristics of immobilized LOA

The capability of a LOA affinity column to bind various polysaccharides and glycoproteins was also investigated. Yeast mannan bound strongly to the LOA column and was

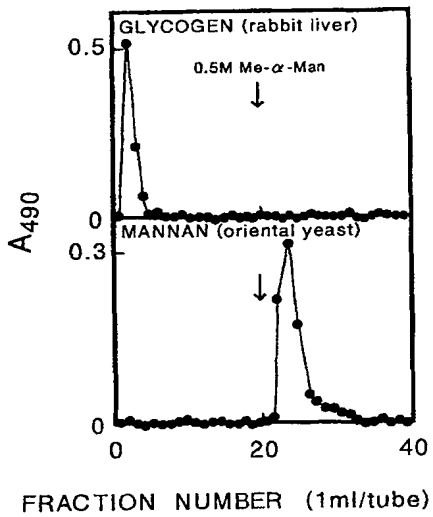

Fig. 3. Elution profile of mannan and glycogen on a LOA-Toyopearl column (1 cm×1 cm). Rabbit liver glycogen (1 mg) was applied to the LOA-Toyopearl column; also shown, *C. tropicalis* mannan (250 μg) was applied to the same column. The arrows indicate the addition of 0.5 mM methyl α-D-mannoside. The methyl glycoside in each fraction was removed by dialysis before assay for total carbohydrate.

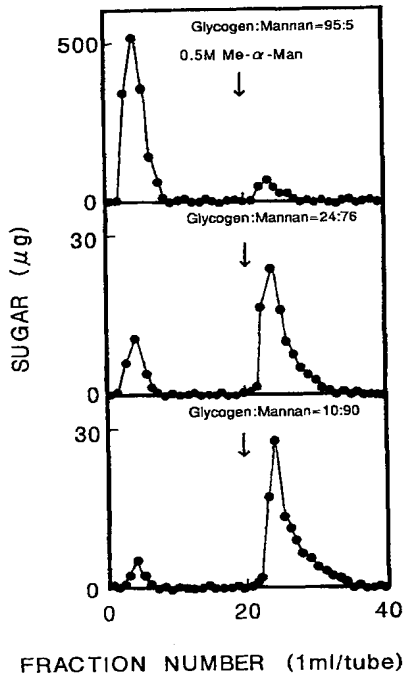

Fig. 4. Elution profiles of yeast polysaccharides containing mannan and glycogen on LOA-Toyopearl. Conditions were similar to those of Fig. 3.

completely eluted with methyl α-D-mannoside, whereas glycogen readily passed through the column unretarded (Fig. 3). When the yeast mannan fraction prepared from *S. cerevisiae* cells, which is usually contaminated with glycogen, was applied to the LOA column, glycogen was resolved completely from the mannan, which was eluted subsequently with 0.5 mM methyl α-D-mannoside, as shown in Fig. 4. Similar results were obtained in three experiments using yeast mannan fractions containing varying proportions of glycogen or cell wall glucans (Fig. 4). This represents a dramatic example of the utility of immobilized LOA for the separation of α-mannans from α-glucans.

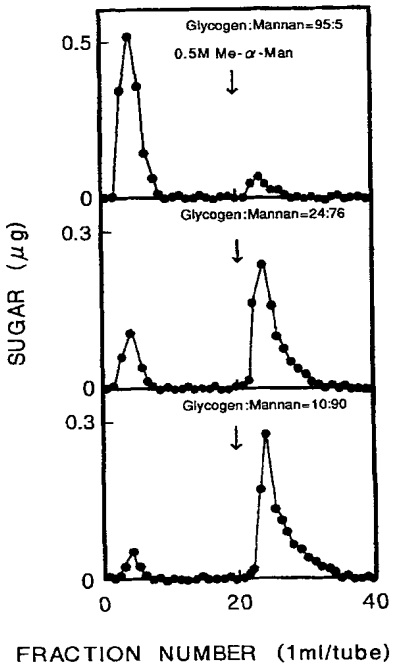

Fig. 5. Elution profiles of some glycoproteins on a LOA-Toyopearl column. Various glycoproteins were applied to a Toyopearl column (1 cm×1 cm), followed by elution with NaCl/P_i and NaCl/P_i containing 0.5 M Me-α-D-Man.

In another experiment, some glycoproteins containing various high levels of α-mannosyl units in their carbohydrate chains were applied to the LOA-Toyopearl column and eluted with NaCl/P_i and NaCl/P_i containing 0.5 mM Me-α-D-Man*p*. The results are shown in Fig. 5. Lima bean lectin which carries a terminal Man(α1-3)Man unit on both carbohydrate chains [17] bound strongly to this column and was eluted with Me-α-D-Man*p* (NaCl/P_i) whereas the *Phaseolus vulgarus* lectin was resolved into two components signifying heterogeneity of the glycosyl moiety [18]. However, ovalbumin, which contains a large number and variety of high-mannose and hybrid-type glycosyl moieties, was not retained on the column as was the case with the immobilized-snowdrop-lectin column [1]. This may be due either to the presences of a single carbohydrate chain, or the location of the (α1-3)-mannosyl sequences on the molecule.

We believe the orchid twayblade lectin should be of great utility for the separation of α-mannans from α-glucans, and for investigating the structure of complex carbohydrates, especially those carrying α1-3-mannosidic linkages.

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